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ORIGINAL ARTICLE

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Strawberry fruit protein with a novel indole-acyl modification

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Abstract Achenes and receptacle tissue of *Fragaria vesca*, L. cultivar Yellow Wonder were shown to contain conjugated indole-3-acetic acid (IAA) that was not soluble in organic solvents and yielded IAA after strong alkaline hydrolysis, suggestive of IAA attached to plant proteins. This solvent insoluble conjugated IAA accounted for between 0.4 and 4 ng of IAA per gram fresh weight of tissue in both achenes and receptacles. To investigate this strawberry conjugate class further, a polyclonal antibody was produced to IAA–glycine attached to BSA that detected neutral indole acid esters, monocarboxylic-amino acid IAA conjugates and IAA proteins. Using immunoblotting, both achenes and receptacles of strawberry were shown to have primarily an immuno-detectable band at 76 kDa. Two-dimensional polyacrylamide gel electrophoresis yielded a wide band that was analyzed by LC–MS/MS analysis following in-gel trypsin digestion. Peptides derived from the immuno-detectable band were tentatively identified by peptide fragment analysis as being from either a chaperonin related to the hsp60 class of proteins or, alternatively, an ATP synthase. This is one of the first reports of an IAA modified protein in fruit tissue.

Keywords ATP synthase · Auxin conjugation · Chaperonin · *Fragaria vesca* · Heat stress protein · IAA protein

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Introduction

Fruit development and ripening correspond to a series of genetically programmed developmental events regulated by endogenous phytohormones and external factors such as light and temperature. For climacteric fruits, research using physiological, biochemical, genetic and reverse genetics approaches has clearly demonstrated the critical role of the plant hormone ethylene in initiating and regulating events associated with ripening. Additional studies documenting changes in hormone metabolism (Epstein et al. 2002), or changes in the levels of plant hormones (Buta and Spaulding 1994), combined with investigations of the effects of hormone-related genetic transformation (Jones et al. 2002) or of the consequences of hormone treatments (Cohen 1996), support the dynamic involvement of multiple hormones in this developmental pathway in tomato. Recent studies of several auxin transcription factor homologs indicated their role in crosstalk between ethylene and auxin during fruit development (Swarup et al. 2002). Down-regulation of one of these (DR12) in tomato resulted in a pleiotropic phenotype including dark-green and blotchy ripening fruit, enhanced firmness, and increased pigment accumulation at the red-ripe stage (Jones et al. 2002).

While several plant systems have been used to study hormonal relationships in fruit development and ripening in climacteric fruit, strawberry receptacles have

specific advantages for such studies in a non-climacteric fruit, and there exists a long history of research on this fruit. Central to growth and ripening in the strawberry is the production of auxin in the achenes, and the dependence of the receptacle on auxin for growth (Nitsch 1950). The fleshy part of the strawberry is derived by the enlargement of the receptacle. The receptacle forms a domed- or cone-shaped projection in the middle of the flower, and the pistils, which become the achenes, cover the outside of the structure. Upon fertilization, the ovule begins to produce auxin, which causes the receptacle to enlarge. Upon cessation of auxin production by the achenes, the receptacle begins to turn red and ripen (Nitsch 1950; Given et al. 1988). As in the climacteric fruit, tomato (Cohen 1996), auxin delays ripening in strawberry at later stages of development (Given et al. 1988).

A number of genes involved in the physiological events of strawberry fruit growth and ripening have been shown to be auxin regulated (Reddy and Poovaiah 1987; Manning 1994, 1998; Civello et al. 1999; Aharoni et al. 2002; Castillejo et al. 2004); however, relatively little is known about how the metabolism of auxin is regulated in this fruit. Several studies have measured free auxin or free and conjugated auxin levels in strawberry fruit tissue during fruit development (Nitsch 1955; Archbold and Dennis 1984; Archbold and Dennis 1985; Hou and Huang 2004) but the regulation of interconversion between these forms, which has potential to regulate fruit size and quality, has not been investigated.

Auxin metabolism in all plants that have been studied in detail involves not only the biosynthesis and degradation of the free hormone, but also the process of conjugate formation and hydrolysis (Woodward and Bartel 2005). Adding to the complexity of plant auxin metabolism is the fact that multiple biosynthetic pathways are known to be present in the same plant and under developmental and environmental control (Michalczuk et al. 1992; Epstein et al. 2002; Rapparini et al. 2002; Sztein et al. 2002). In addition, conjugated forms of indole-3–acetic acid (IAA) are present in plant tissues and can be small molecular weight compounds where IAA is linked via ester or amide bonds (Cohen and Bandurski 1982) or higher molecular weight conjugates such as small peptides, protein, glycans and glycoproteins. Strawberry is known to contain both extractable ester and amide IAA conjugates (Archbold and Dennis 1984), but only IAA-glucose has been identified (Wawrzyńczak et al. 2000). Of particular interest to us have been the protein conjugates for which IAA appears to serve as a post-translational modifier (Walz et al. 2002), consistent with the observation that there are no measurable levels of IAA derivatized free amino acids in bean (Bialek and Cohen, 1986). We now report that strawberry fruit, a classic system for studies of auxin regulation of fruit development, contains an IAA-modified protein as an important component of its conjugate pool.

Materials and methods

Plant material

Seeds of *Fragaria vesca* spp. *vesca* f. *semperflorens* cultivar Yellow Wonder from the collection at USDA/ARS in Beltsville, MD were sown in Metro-Mix 510 (Sun-Gro, Seba Beach, Alberta, Canada). Plants were cultivated in the greenhouse in Beltsville, MD under a 12 h photoperiod supplemented with Na-halide lamps. Plants were fertilized bimonthly with Miracle-Gro Tomato Plant Food (18-18-21) (Scotts Miracle-Gro Products, Inc., Marysville, OH) according to the manufacturers directions. Primary or secondary fruit were harvested from the primary inflorescence, weighed, and either immediately frozen in liquid N_2 or rapidly separated into receptacles and achenes, which were then separately frozen in liquid N_2 after weighing, shipped on dry ice to the University of Minnesota, and stored at −80°C until analysis.

Preparation of IAA–glycine immunogen

IAA–glycine was linked to bovine serum albumin using methods similar to those previously described (Yamaguchi et al. 1987) and using the suggested improvements for water-based coupling (Sehgal and Vijay 1994). IAA–glycine (5.2 mg, 22.4 µmol, Research Organics, Cleveland OH) and *N*-hydroxysuccinimide (4 mg, 34.8 µmol, Pierce, Rockford, IL) were dissolved in 0.8 ml dimethylformamide (DMF). An excess of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC; 134.2 mg, 700 µmol, Pierce) in 3.9 ml of 0.1 mM 2-morpholinoethanesulfonic acid buffer, pH 5.0 was added and the reaction mixture incubated in 4°C for 100 min. This reaction mixture was added to bovine serum albumin (40 mg, 0.6 µmol, Sigma, St. Louis, MO) dissolved in 2 ml of 0.1 mM 2-morpholinoethanesulfonic acid (MES) buffer, pH 5.0. The reaction was continued for 24 h at 4°C and the coupled protein product was dialyzed through six sequential steps with: (1) 10% DMF in 10 mM MES pH 5.0; (2) 10% DMF in 10 mM MES pH 5.0; (3) 5% DMF in 10 mM MES pH 5.0; (4) Phosphate buffered saline (PBS; 137 mM sodium chloride, 5 mM sodium phosphate, pH 7.4.); (5) half-strength PBS; (6) Distilled water. Each dialysis step was against 1 l of solution for 12 h and all steps were carried out in a 4°C coldroom. MALDI-TOF mass spectra of the conjugated immunogen were obtained on a Bruker Daltonics Biflex III instrument equipped with an N_2 laser, using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) as the matrix, and unreacted bovine serum albumin for calibration, as previously described for a related immunogen (Ilic et al. 2005). Antibodies were generated (Sigma Genosys custom antisera, http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Antibody_Explorer/Custom_Antiseraa.html) by subcutaneous injection into two New Zealand white rabbits. Prior to the first injection, a

pre-immune bleed was collected, followed by a 77-day schedule that included six immunizations per animal and four bleeds per animal. The initial immunization was given in complete Freund's adjuvant and all subsequent immunizations in incomplete Freund's adjuvant. The antibody derived from this procedure is referred to as AbIAA–G–BSA and was tested for specificity to several indolic compounds by an enzyme-linked immunosorbent assay, essentially as described previously (Ilic et al. 2005) except that the tracer was IAA–glycine linked to alkaline phosphatase (calf intestine, #0108138; Roche Applied Science, Indianapolis, IN). Microtiter plates were treated with goat antirabbit FC-fragment (#401963; Calbiochem, San Diego, CA) before adding the AbIAA–G–BSA. The tracer was reconstituted at 0.5 mg/ml and a 4,000× dilution of this was used. Compounds analyzed were tested at a range of concentrations from 1.0 pM to 10 nM.

Immunoblotting

Achenes and receptacles from immature fruit of strawberry (*F. vesca*) and seeds from pea (*Pisum sativum*, line I, Alaska-type) were frozen in liquid N_2 and ground to a powder in a liquid N_2 frozen mortar and pestle (500 ml). Dry seeds of Tepary bean (*Phaseolus acutifolius*), bush bean (*Phaseolus vulgaris*, Burpee bush stringless), and *Arabidopsis thaliana* (Columbia) were ground in a mortar and pestle without freezing. The ground materials were washed in a filter funnel (Whatman #1 paper) with acetone at −20°C and the powder air-dried to remove residual solvent. Proteins were extracted from the acetone powders with Laemmli buffer (Laemmli, 1970) and separated on a 10% denaturing polyacrylamide gel. The proteins were blotted for 75 min at 0.8 mA/cm^2 onto nitrocellulose (BioRad) membranes. The membranes were blocked with 5% (w/v) non fat dried milk in TBS buffer followed by incubation with preimmune serum at 1:1,000 dilution, antibody Ab3.6 K raised against the bean 3.6 kDa IAA peptide (Walz et al. 2002) at 1:1,000 dilution or with AbIAA–G–BSA at 1:1,000 dilution. Detection was done according to the manufacturer's instructions using the immun-blot goat anti-rabbit IgG (H + L)-alkaline phosphatase assay kit (#170-6460, Bio-Rad), which is based on a nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate colorimetric substrate system.

Analysis of strawberry protein with IAA attachment

Total protein was extracted from strawberry achenes with buffer (2% CHAPS, 5% beta-mercaptoethanol, 40 mM Tris–HCl) containing 7 M urea and 2 M thiourea. A sample containing 50 µg of protein was loaded onto the first-dimension isoelectric focusing system (pH 3-10 strip, 7 cm, #163-2000, BioRad), followed by SDS-PAGE. The AbIAA–G–BSA detected protein spot on a 2D-PAGE gel was determined by locating the zone of immuno reactivity on the membrane blot and matching with the Coomassie Blue staining. The wide spot on the gel was cut into four spots of increasing pI and analyzed individually following in-gel trypsin digestion (Shevchenko et al. 1996). Samples were analyzed using an ESI source on an ABI (Applied Biosystems, Foster City, CA) QTRAP triple quadrupole with Q3 Linear Trap MS with an Agilent capHPLC 1100 running a C_{18} capillary (0.3 mm × 100 mm) and an acetonitrile/water gradient. MS-MS spectra were matched with NCBI's (National Center for Biotechnology Information, http://www.ncbi.nlm. nih.gov/) non-redundant (NR) protein database (Wheeler et al. 2001) using Pro ID (ABI) software. Since there is only limited strawberry protein database information, each spectrum was matched to the closest peptide from all available plant species.

Quantitative analysis of IAA-protein content

The extracted total protein, after extensive acetone washing, was hydrolyzed in 7 N NaOH and purified on a Baker C_{18} solid phase extraction column prior to GC-MS analysis (Chen et al. 1988). [$^{13}C_6$]IAA was used as internal standard (Cohen et al. 1986) and samples were methylated with diazomethane (Cohen 1984) prior to injection onto the gas chromatograph. Analysis was performed by GC-MS (Model 6890 GC/5973 MSD, Agilent Technologies, Palo Alto, CA). The molecular and quinolinium ions for methyl-IAA at m/z 189/195 and 130/136, respectively, were monitored (ions deriving from the methyl esters of endogenous and [$^{13}C_6$]IAA, respectively). The amount of IAA released by alkaline hydrolysis of the protein was calculated by the isotope dilution equation.

Results

IAA–glycine linkage to BSA was studied by MALDI-MS analysis (data not shown) which confirmed that between 7 and 10 IAA–glycine residues were linked to each BSA molecule, within the range recommended for antigens (Landsteiner 1990). The antibody produced interacted strongly, as expected, to IAA–glycine and less strongly to a range of neutral esters of indolic acids (Table 1). Little or no affinity was seen with free tryptophan and the interaction with free IAA and the methyl ester of phenylacetic acid were both very weak. Previous studies of IAA proteins relied on an antibody, Ab3.6 K, generated from a small 3.6 kDa IAA peptide isolated from bean (Bialek and Cohen 1986; Walz et al. 2002). However, as shown in Fig. 1, the Ab3.6 K cross-reacted with only a subset of IAA proteins, presumably those related to IAP1 (Walz et al. 2002), in several plant species. Some plant proteins were not as well detected by Ab3.6 K antibody as by AbIAA–G–BSA. The bean IAP1 protein [a 35 kDa protein that runs anomalously high on SDS-PAGE gels (Walz et al. 2002)], both from *Phaseolus vulgaris* and *Phaseolus acutifolius* (Tepary bean), was detected by both

Table 1 Specificity of the AbIAA–G–BSA antibody to several related indolic compounds determined using an enzyme-linked immunosorbent assay

Compound	Relative detection
L-Tryptophan	< 0.001
Phenylacetic acid, methyl ester	0.004
Indole-3-butyric acid, methyl ester	17.439
Indole-3-acetic acid, methyl ester	19.471
Indole-3-acetyl-ε-*N*-L-lysine	21.110
Indole-3-acetyl-glycine	100.000
Indole-3-acetyl-L-aspartate	0.578
Indole-3-acetic acid	0.055
4-Cl-indole-3-acetic acid, methyl ester	14.361
4-Cl-indole-3-acetyl-L-aspartate	0.423

The tracer was IAA–glycine linked to alkaline phosphatase. Compounds analyzed were tested at a range of concentrations from 1.0 pM to 10 nM and the concentration necessary to give a 50% reduction in enzyme response is expressed relative to the concentration required to reduce the response by 50% when using IAA–glycine

Fig. 2 Immunoblot analysis of 50 µg of strawberry achene proteins resolved by two-dimensional polyacrylamide gel electrophoresis. The IAA proteins were detected using the AbIAA–G–BSA antibody

antibodies. The strawberry IAA proteins, which by 1D PAGE appear as a doublet (Fig. 1) but more typically appear as a single band (Fig. 3) at 76 kDa, were further resolved on 2D PAGE as a broad band spanning almost 1.0 pI (Fig. 2). Protein at the same molecular mass was detected by AbIAA–G–BSA in extracts from mature achenes or from fruit tissue (Fig. 3).

The levels of protein-linked IAA were determined in the cold acetone insoluble total protein extract from whole fruit, receptacles without achenes, and in the mature achenes. The level of IAA in protein in the receptacles of immature fruit $\leq$ 1 cm in length and in mature but unripe fruit 1–2 cm in size was 0.55 ± 0.14 ng/mg fresh weight. In contrast, the level of IAA in protein in whole fruit increased tenfold, from 0.41 to 4.0 ng/mg, as the fruit increased in size. Surprisingly, fully mature achenes contained 1.1 ng/mg protein-bound IAA, somewhat less on a fresh weight basis than whole fruit at the 1–2 cm size. This discrepancy may be due to the density of the seed coat.

Identification of the IAA-protein was attempted using LC–MS–MS (Table 2). The spectra obtained from multiple peptides obtained from the 2D-PAGE analysis allowed tentative assignment to two general classes of proteins with scores of > 99% found in higher plants, HSP/Chaperonin 60-like proteins and an ATP synthase. Because no peptide with adequate ionization yielded a strong IAA–peptide m/z 130 quinolinium signature ion (Park et al. 2004), final assignment of protein identity containing the IAA moiety will require further mass spectral analysis to obtain spectra on a more complete set of peptides.

Discussion

In order to efficiently detect IAA linked to plant proteins, it was desirable to develop a suitable antibody with

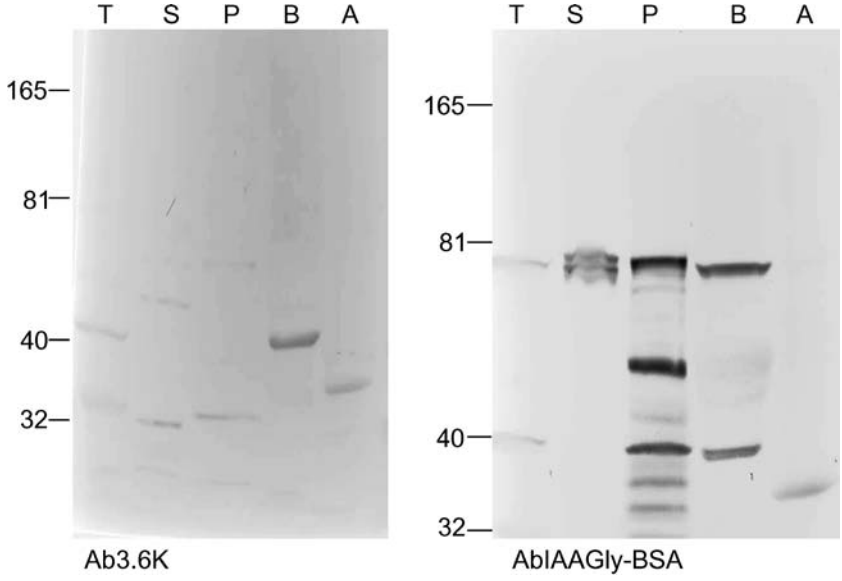

Fig. 1 Immunoblot analysis comparing proteins detected using an antibody prepared using the bean 3.6 kDa IAA–peptide as the immunogen (Ab3.6K, *left gel blot*) to the one detected using the antibody obtained when rabbits were injected with IAA–glycine–BSA (AbIAA–G–BSA, *right gel blot*). *Lanes* were loaded with 30 µg of protein extracts prepared from seeds of *Phaseolus acutifolius* ("T", Tepary bean), *Fragaria vesca* ("S", strawberry), *Pisum sativum* ("P", pea), *Phaseolus vulgaris* ("B", bush bean) and *Arabidopsis thaliana* ("A"), prepared as described in Materials and methods

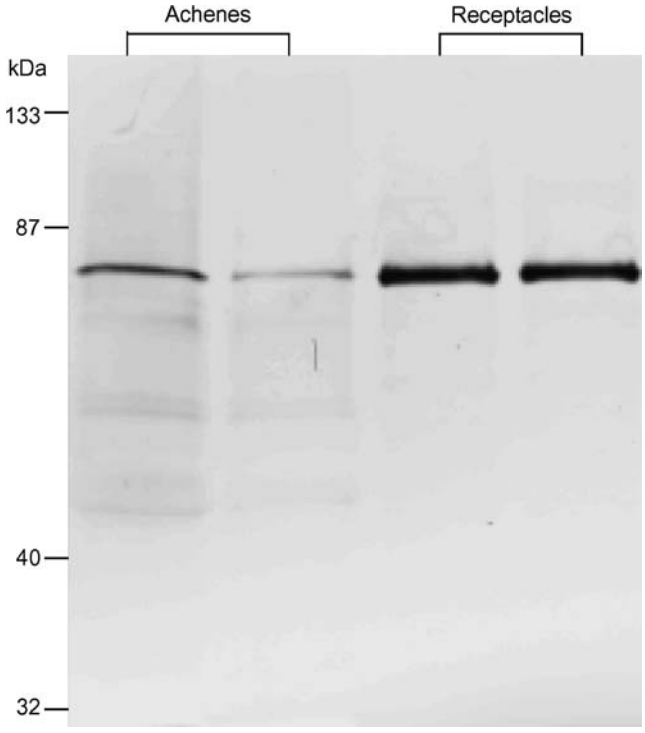

Fig. 3 Immunoblot analysis of strawberry achene and receptacle proteins following polyacrylamide gel electrophoresis. *Lanes* were loaded with approximately 30 μg protein. The IAA proteins were detected using the AbIAA–G–BSA antibody

the required affinity and specificity for immunoblots. Antibodies elicited using IAA linked directly to a carrier protein through the carboxyl have been most commonly used for immunohistochemistry (Avsian-Kretchmer et al. 2002; Thomas et al. 2002; Hou and Huang 2004, 2005), and were purported to detect IAA linked to proteins. In our hands, such antibodies gave equivocal results when tested with known IAA proteins, including bean IAP1 protein and *Arabidopsis* IAA–peptides (data not shown). This result could explain the lack of response noted with tissue not treated with cross-linking agents reported in the previous work. Earlier work by our group employed an antibody, Ab3.6 K, obtained by the injection of a 3.6 kDa peptide into rabbits without linkage to any larger protein. This peptide was effective at identification of a number of IAA proteins in several plant species, and was used by us for a limited tissue printing study in melon and tomato fruit (Cohen et al. 1995); however in some cases, it detected proteins with little or no IAA attached (data not shown). Therefore, we felt it necessary to design an antibody that would more effectively identify indoleacyl-modified proteins in plants. As shown in Fig. 4, adding a glycine spacer between IAA and BSA allows better access to the IAA moiety, increasing the potential for elicitation of an antibody with greater specificity for the indole-acyl modification on the protein core. Consistent with this expectation, the resulting antibody, AbIAA–G–BSA, showed high specificity for neutral indolic esters and IAA–glycine itself. Aspartate conjugates between IAA or 4-Cl-IAA were poorly recognized (Table 1) and this may be due to the highly polar nature of the dicarboxylic acid conjugates, or to the fact that the amino acid backbone of dicarboxylic acid conjugates bend over the indole nucleus in a hair-pin conformation (Antolic et al. 2001), possibly shielding them from recognition by the antibody. Ab3.6 K and AbIAA–G–BSA detect IAP1 from bean and Tepary bean on immunoblots, and IAA modified proteins from pea and *Arabidopsis* are also detected by both antibodies (Fig. 1). Analysis of the pea proteins detected by AbIAA–G–BSA showed that some have IAA and others have 4-Cl-IAA attached in amide linkage (Ozga and Park, unpublished data).

Proteins from strawberry recepticles and achenes contain IAA that is not extracted into organic solvents, but which is released by strong alkaline hydrolysis. This conjugated IAA accounts for between 0.4 and 4 ng of IAA per mg of tissue. This level of IAA in the IAA-protein fraction was significantly greater than the amount of acetone-soluble IAA amide conjugate, most likely IAA-amino acids, previously found in recepticle tissues (estimated from the data in Archbold and Dennis (1984) to be 0–50 pg/mg fresh weight). The protein conjugated IAA values are comparable to the values that were reported previously for free IAA (0.2–3 ng/mg) and acetone extractable IAA amide conjugates (1.0–28 ng/mg) in strawberry achenes (Dreher and Poovaiah 1982; Archbold and Dennis 1984; Archbold and Dennis 1985), indicative of its potential to be an important part of the auxin economy in this plant.

Both receptacles and achenes contain solvent-insoluble IAA conjugates. In both tissues, a 76 kDa band is detected on the immunoblots of 1D gels (Fig. 3), and this 76 kDa band appears as a broad band on 2D gels (Fig. 2) indicating heterogeneity in the isoelectric point. This isoelectric point behavior could be due to variations in the number of sites modified by IAA or other moieties, to other structural features, or to more than one closely related protein being present. Analysis of the peptides resulting from tryptic digestion of several regions of this broad band suggests that the same, or similar protein sequences, are present throughout (Table 2). The apparent molecular weight of the proteins on gels appears to be somewhat higher than that predicted based on the proteins identified by peptide analysis (60–63 kDa, Table 2), however IAP1 from bean also displayed an anomalous behavior on protein sizing gels, where gene sequence and MALDI-MS identified a 35 kDa protein that by electrophoresis ran as if its molecular mass was 42 kDa (Walz et al. 2002).

Knowledge about the presence of IAA-modified proteins in fruit and achenes of strawberry has the potential to help solve questions about the auxin relationships in these tissues, and sheds light on a number of questions raised in earlier studies about the highly variable levels of IAA and known conjugates found during strawberry development (Nitsch 1955; Archbold and Dennis 1984,

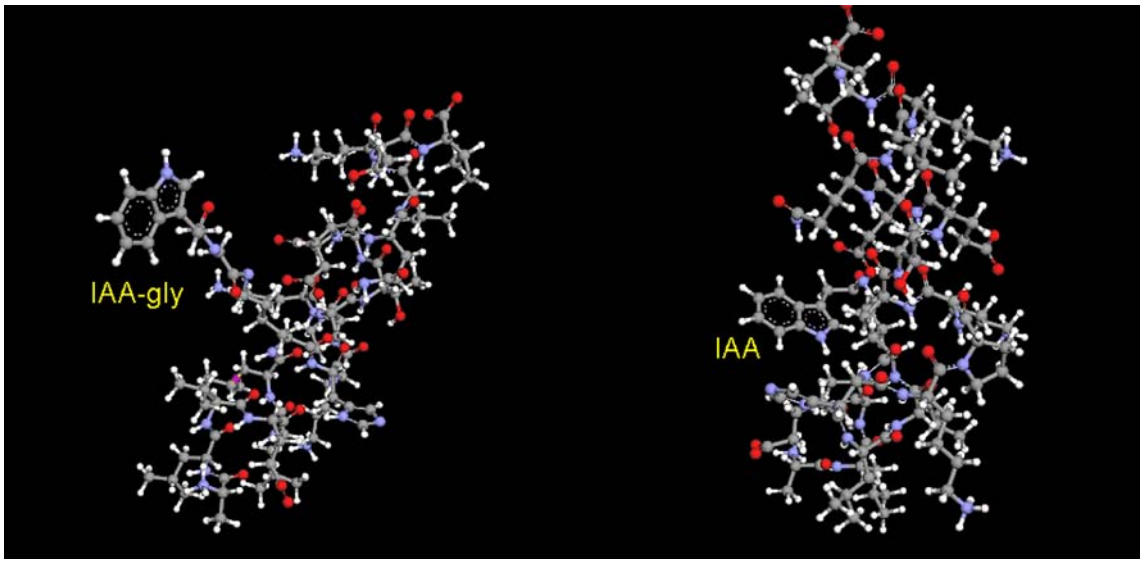

Fig. 4 Computational analysis of IAA–glycine or IAA linked to a lysine moiety of a BSA peptide fragment (ALVELLKHKPKATEE QLKTVMEN) to illustrate the approximate geometry resulting from the two linking strategies. The image was developed using ArgusLab 4.0.1 software (Planaria Software LLC, Seattle, WA) using > 10,000 iterations each for generation of the two images with optimized geometry

Table 2 Summary of assignments of protein identity based on peptide scores following LC–MS–MS analysis of four spots from 2D PAGE identified based on immunological detection using AbIAA–G–BSA

Spot no.	Name	Species	Accession
1	Chaperonin precursor	*Pisum sativum*	AAA66365.1
	ATP synthase	*Triticum aestivum*	CAA52636.1
	Heat shock protein 60	*Prunus dulcis*	AAN63805.1
2	Heat shock protein 60	*Prunus dulcis*	AAN63805.1
	Chaperonin 60	*Solanum tuberosum*	AAB39827.1
	ATP synthase	*Triticum aestivum*	CAA52636.1
3	Chaperonin precursor	*Pisum sativum*	AAA66365.1
	ATP synthase	*Triticum aestivum*	CAA52636.1
	Heat shock protein 60	*Prunus dulcis*	AAN63805.1
4	No proteins identified at > 95%		

MS–MS spectra were matched with NCBI's NR protein database using Pro ID (ABI) software. Only limited strawberry protein database information is currently available, thus each peptide spectrum was matched to sequences from all available plant species

1985; Hou and Huang 2004). Our analyses also open up new questions concerning the use of immunohistochemical techniques for auxin detection. As shown, a significant amount of the IAA in strawberry fruit tissues is linked to solvent insoluble proteins. Nevertheless, recent reports where IAA is purported to be immunolocalized in strawberry tissue prefixed with EDC (Hou and Huang 2004, 2005) failed to detect IAA in control tissues, which while not being prefixed with EDC, should have contained IAA linked to solvent insoluble protein. It therefore remains to be determined if such immunolocalization methods are detecting IAA or IAA-amino acids randomly linked by EDC to cell proteins. It is also important to determine why such methods failed to detect endogenous proteins with an IAA moiety in the control, non-prefixed samples. As shown in Fig. 1, immunodetection of IAA modified proteins is not limited to strawberry, and thus efforts using immunohistochemistry to localize IAA in other species (Avsian-Kretchmer et al. 2002; Thomas et al. 2002) need further clarification.

Finally, IAP1, the IAA protein identified from bean, is a late embryo abundant protein presumably involved in storage with no other known function. Although the identity of the strawberry protein has not been fully resolved in this study, the possibility that IAA is linked to proteins with chaperone or enzyme activity in actively growing tissues opens up new possiblities for future studies on the biological significance of hormone modification of cell proteins. A future area we plan to investigate is the relationship between stress effects on IAA metabolism (reviewed in Ljung et al. 2002) and the activity of the heat regulated proteins, ATP synthase (Majoul et al. 2004), chaperonin, and heat shock 60 protein (Baniwal et al. 2004), identified here as candidates for indole-acyl modification in the strawberry.

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